

## Stimulation of exopolysaccharide production by fluorescent pseudomonads in sucrose media due to dehydration and increased osmolarity

---

### Abstract

Exopolysaccharides produced by plant pathogenic bacteria are thought to play an important role in both the general ecology and the virulence of the producing organism. The environmental factors affecting exopolysaccharide production in planta by *Pseudomonas syringae* pathovars are not known. We tested the effect of increased medium osmolarity and dehydration on exopolysaccharide production in a sucrose-containing medium by three *P. syringae* pathovars, one (*P. syringae* pv. *phaseolicola*) capable of levan and alginate production and two (*P. syringae* pv. *papulans* and pv. *savastanoi*) capable of only alginate production. Addition of NaCl and ethanol to the medium led to increased accumulation of alginate by all three pathovars as well as increased levan production by *P. syringae* pv. *phaseolicola*. Culture fluids of the two non-levan producers also contained increased amounts of neutral carbohydrate which was not levan. Based on sugar composition this material may have originated from outer membrane lipopolysaccharide. In addition, the ratio of neutral material (levan or not) to alginate varied dependent on culture conditions.

### 1. Introduction

Production of exopolysaccharide (EPS) by a wide range of bacteria including plant pathogens has been well documented [1,2]. These polymers may mediate adhesion to living and nonliving surfaces, protect against adverse environmental conditions such as dehydration, concentrate bacterial enzymes and required cations at the bacterial cell surface and act as

virulence factors for human, animal and plant pathogens [3–5].

The important plant pathogen *Pseudomonas syringae*, which usually induces lesions on leaves of host plants, is further separated into pathovars based primarily on host range and/or types of symptoms they cause [6]. In vitro, *Pseudomonas syringae* pathovars are able to produce the acidic polymer alginate 1,4-linked linear co-polymer of  $\beta$ -D-mannuronic acid and  $\alpha$ -L-guluronic acid [7]. Some strains also are capable of producing the neutral EPS levan (a polyfructan) [8]. Levan is produced only when sucrose is available through the action of the extra-

cellular enzyme levansucrase. When high levels (2.4%) of sucrose were added to the growth medium, *P. syringae* pathovars produced either levan or alginate alone or produced both together [7,8]. However, alginate is preferentially produced in the leaves of plants infected by *P. syringae* pathovars, both by pathovars which are capable of levan production in vitro and those that are not, though sucrose was the main plant host translocatable sugar [9,10], factors which regulate EPS production by these bacteria in the plant leaf are not known.

In a previous study, we demonstrated that alginate production by some, but not all, plant-associated fluorescent pseudomonads was increased by high osmolarity (0.2–0.5 M sodium chloride) and by dehydration (1–3% ethanol) in a complex broth medium with glycerol as the primary carbon source [11]. Previous to our studies with plant pathogenic and plant-associated pseudomonads, stress brought on by a hyperosmotic environment and dehydration was reported to increase both transcription of alginate structural and regulatory genes and alginate production in *P. aeruginosa* (for review see [12]).

As *P. aeruginosa* is not capable of levan production, it was of interest to study the effects of these two environmental parameters for *P. syringae* pathovars under cultural conditions which allowed for production of levan. Since sucrose is most likely the primary carbon source in leaf apoplastic fluids of many host plants we wished to determine if stress induced by dehydration and hyperosmolarity leads to increased alginate and levan production in a sucrose-containing medium and if such stresses would alter the relative amounts of alginate and levan produced.

## 2. Materials and methods

### 2.1. Bacterial strains and growth conditions

Strains representing three *Pseudomonas syringae* pathovars were utilized: *P. syringae* pv. *phaseolicola* Race 2 (originally isolated from *Phaseolus vulgaris* and obtained from D.M. Webster), *P. syringae* pv. *savastanoi* GTG24 (originally isolated from a *Jasminum* sp. and obtained from G. Surico), and *P. syringae* pv. *papulans* (originally isolated

from *Malus sylvestris* and obtained from T.J. Burr). The strains were maintained on *Pseudomonas* agar F medium (Difco Laboratories) at 4°C.

Bacteria were cultured in a semisynthetic liquid medium described by Bruegger and Keen [13] with potassium phosphate substituted for yeast extract and with sucrose (50 g/l) as the carbon source. Starter cultures were incubated overnight and 2 ml was used to inoculate 25 ml of broth contained in 250 ml Erlenmeyer flasks. Flasks were shaken (200 rpm) for 24–30 h at 28°C at which time control cultures (no added sodium chloride or ethanol) had reached stationary phase based on cell dry weight measurements.

### 2.2. Isolation and purification of exopolysaccharides

After incubation, cells were harvested by centrifugation ( $7800 \times g$ , 15 min) in preweighed centrifuge tubes and the supernatant fluids collected. The cells were washed once with 5 ml of sterile water, and the final cell pellets were dried to a constant weight at 80°C. The culture supernatant fluids plus the cell washes were combined and subjected to precipitation with ice-cold ethanol (3–4 volumes) after addition of KCl to a final concentration of 1% (w/v). After 20 minutes of stirring, the resultant precipitate was collected by centrifugation ( $17000 \times g$ , 15 min), suspended in 5 ml of distilled water, and vigorously mixed for 2 min. Any insoluble material was then removed from the samples by centrifugation ( $17000 \times g$ , 15 min).

Ion-exchange chromatography was utilized for the separation of anionic and non-anionic exopolysaccharides. Samples were loaded onto a column of DEAE-Sepharose CL-6B (Pharmacia) equilibrated with 0.05 M Tris · HCl buffer, pH 8.0. Subsequently the column was washed with three bed volumes of buffer alone. After collecting the buffer eluate the column was eluted with buffer containing 1 M NaCl. The unretained (non-acidic) and retained (acidic) fractions were dialyzed against distilled water, dried under vacuum and weighed.

### 2.3. Characterization of exopolysaccharides

The amount of the alginate in each sample was determined by use of a colorimetric assay for uronic

acid as described by Blumenkrantz and Asboe-Hansen [14] using a commercial sample of algal alginate (Sigma) as the standard.

The amount of non-acidic exopolysaccharides present in the samples which were eluted from the anion exchange column with buffer alone was determined after dialysis and lyophilization by the colorimetric method of Dubois et al. [15] with D-glucose (Sigma) as the standard. The amount of levan in these samples was estimated by a colorimetric assay for detection of ketohexoses as described by Ashwell [16] with D-fructose (Sigma) as standard. The presence of levan was confirmed in selected samples by GLC analysis. Samples were hydrolyzed with 1 M oxalic acid (70°C, 1.5 h) and the released sugars were identified as their acetate derivatives [9]. Analysis by GLC was performed using a Hewlett-Packard model 5995B gas-liquid chromatograph fitted with a SP-2330 capillary column (15-M) (Supelco) with temperature programming from 150°C to 250°C at 4°C/min. Chromatograms were compared to those

obtained for a levan standard (Sigma). Putative levan samples were also hydrolyzed with either 1 N H<sub>2</sub>SO<sub>4</sub> (100°C, 1.5 h) or 2 M trifluoroacetic acid (120°C, 1 h), and the released sugars identified as their aldononitrile acetate derivatives [17] by GLC as described above to determine if samples contained sugars other than fructose.

### 3. Results and discussion

#### 3.1. Effects of increased osmolarity by addition of sodium chloride

Growth of all three strains was inhibited by addition of sodium chloride with inhibition increasing along with the levels of NaCl present (Fig. 1). At the highest level of NaCl (0.7%), inhibition ranged from 52 to 74%. All strains produced detectable amounts of alginate in sucrose-containing broth medium with no sodium chloride added (Fig. 1). Addition of NaCl

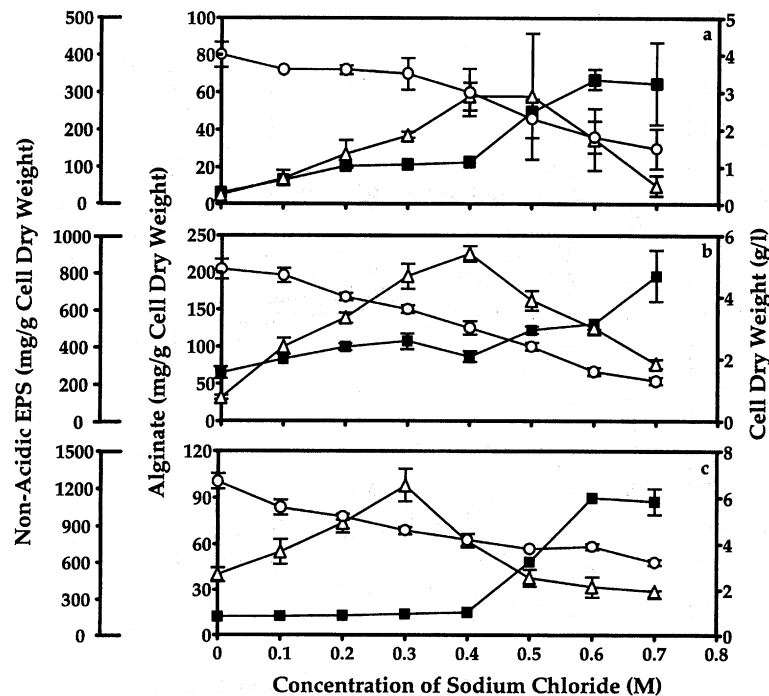


Fig. 1. Effect of addition of sodium chloride to sucrose-containing media on production of acidic (alginate) (△) and non-acidic (■) exopolysaccharide (EPS) and final cell dry weights attained (○). (a) *P. syringae* pv. *papulans*; (b) *P. syringae* pv. *phaseolicola*; and (c) *P. syringae* pv. *savastanoi*. Values are means of values from two experiments ± standard deviations.

to the medium caused increased production of alginate with optimal concentrations ranging from 0.3 to 0.5 M. The greatest stimulation in alginate production (approximately 12-fold) occurred for *P. syringae* pv. *papulans* 17 in the presence of 0.4–0.5 M NaCl (Fig. 1a). These results were similar to those of a previous study done in our laboratory for the plant pathogens *P. syringae* pv. *glycinea*, *P. viridiflava* and the plant-associated saprophyte *P. fluorescens* where up to a 22-fold increase in alginate production (on a cell dry weight basis) was noted [11].

Production of levan by *P. syringae* pv. *phaseolicola* Race 2 was also increased by addition of NaCl to the medium (up to a maximum of 3-fold at 0.7 M NaCl), but to a lesser degree than for alginate (Fig. 1b). Quantitative analysis both by colorimetric assay for ketose sugar and by GLC confirmed that the samples were composed of levan. The ratio of the amounts of levan to alginate produced changed with the amount of NaCl present in the medium. The ratio

gradually decreased from 8:1 for the control cultures to 1.5:1 for cultures containing 0.4 M NaCl. Thereafter the ratio gradually increased to 10:1 at 0.7 M NaCl. Thus, the osmotic environment did effect the nature of the EPS produced. Osmolarity of the culture medium was also reported to affect the relative amounts of two distinct EPSs produced by *Rhizobium meliloti* [18].

The amount of extracellular high molecular mass non-acidic material present in culture supernatant fluids of *P. syringae* pv. *papulans* 17 and *P. syringae* pv. *savastanoi* GTG also increased upon addition of NaCl (Fig. 1a and Fig. 1c) even though these two strains are incapable of levan production. Results of colorimetric assays for ketose sugar and GLC analyses confirmed that no levan was present. A compositional analysis of the samples by GLC after hydrolysis with sulfuric acid and preparation of the aldonitrile acetate derivatives demonstrated the presence of glucose, galactose, ribose, deoxyribose and rhamnose with glucose predominant in all sam-

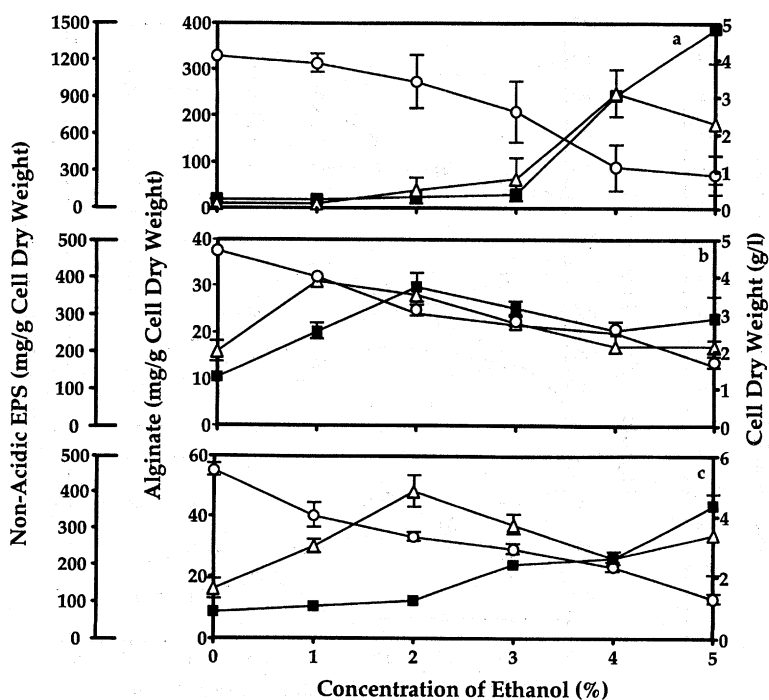


Fig. 2. Effect of addition of ethanol to sucrose-containing media on production of acidic (alginate) ( $\Delta$ ) and non-acidic ( $\blacksquare$ ) exopolysaccharide (EPS) and final cell dry weights attained ( $\circ$ ). (a) *P. syringae* pv. *papulans*; (b) *P. syringae* pv. *phaseolicola*; and (c) *P. syringae* pv. *savastanoi*. Values are means of values from two experiments  $\pm$  standard deviations.

ples based on relative peak areas. Based on the sugar composition, this material may be composed of a mixture of residual nucleic acids remaining after ion-exchange chromatography and neutral carbohydrate oligomers originating from outer membrane lipopolysaccharides [19,20]. It has been reported that at least a portion of the lipopolysaccharides of *P. syringae* pathovars are loosely held and can be extracted by washing with saline [21], but why similar material is not also in the culture supernatant fluids of *P. syringae* pv. *phaseolicola* remains unexplained.

The addition of ethanol to the culture medium led to decreases in growth of all three bacterial strains (Fig. 2) similar to that found for addition of NaCl. Alginate production by the three bacterial strains was also stimulated by the addition of ethanol. The greatest increase (25-fold) in alginate production was found for *P. syringae* pv. *papulans* 17 with ethanol at 4% (Fig. 2a). Greatest stimulation of alginate production for *P. syringae* pv. *phaseolicola* Race 2 occurred with 1% ethanol (2-fold) (Fig. 2b) and for *P. syringae* pv. *savastanoi* with 2% (3-fold) (Fig. 2c).

Levan production by *P. syringae* pv. *phaseolicola* Race 2 was also stimulated in the presence of ethanol with a maximum 3-fold increase in the presence of 2% ethanol (Fig. 2b). Again, colorimetric analyses for ketose sugar as well as GLC analyses indicated the presence of levan alone. In contrast to the results with NaCl, increasing ethanol concentration in the medium above 1% led to an increased ratio of levan to alginate indicating that stress induced by changes in osmolarity and by dehydration may affect the regulatory systems controlling EPS syntheses differently.

As for NaCl, the addition of ethanol stimulated accumulation of high molecular mass, non-acidic material in the culture supernatant fluids of the levan nonproducers *P. syringae* pv. *papulans* and pv. *savastanoi* (Fig. 2a,c). Analyses by colorimetric assay and GLC again indicated that this material might be composed of nucleic acid and lipopolysaccharide oligomers.

In conclusion, this study has demonstrated that production of alginate and levan by the plant pathogen *P. syringae* is stimulated in vitro when stressed by high osmolarity or by dehydration. The

stimulation of alginate production by these two stresses may be due to transcriptional activation of the structural gene *algD* and the global regulatory gene *algR1* as for *P. aeruginosa* [12]. Regulation of a second global regulatory gene (*repA*) affecting alginate production and recently described for the plant pathogen *P. viridiflava* may also be involved [22]. Exopolysaccharide production by *P. cepacia*, a human and plant pathogen, was also recently described to be optimal in the presence of 0.4 M NaCl [23]. Plant pathogenic bacteria contain additional genes called *hrp* genes whose expression is regulated by environmental factors including osmolarity and whose products are also critical factors in host-pathogen interactions [24]). However, the *hrp* genes are repressed under conditions of high osmolarity. Why alginate is preferentially produced in planta is still an open question that requires further study.

## Acknowledgements

We would like to thank T.J. Burr, G. Surico and D.M. Webster for supplying the bacterial strains used in this study. We are also thankful to Mrs. Jane Cochran for typing the manuscript.

## References

- [1] Sutherland, I.W. (1955) Biosynthesis and composition of Gram-negative bacterial extracellular and wall polysaccharides. *Annu. Rev. Microbiol.* 39, 243–270.
- [2] Whitfield, C. (1988) Bacterial extracellular polysaccharides. *Can. J. Microbiol.* 34, 415–420.
- [3] Costerton, J.W., Chen, K.J., Geesey, G.G., Ladd, T.I., Nickel, J.C., Dasgupta, M. and Marie, T.J. (1987) Bacterial biofilms in nature and disease. *Annu. Rev. Microbiol.* 41, 435–464.
- [4] Osphir, T. and Gutnick, D.L. (1994) A role of exopolysaccharides in the protection of microorganisms from dessication. *Appl. Environ. Microbiol.* 60, 740–745.
- [5] Roberson, E.B. and Firestone, M.K. (1992) Relationship between desiccation and exopolysaccharide production in a soil *Pseudomonas* sp. *Appl. Environ. Microbiol.* 58, 1284–1291.
- [6] Young, J.M., Takikawa, Y., Gardan, L. and Stead, D. E. (1992) Changing concepts in the taxonomy of plant pathogenic bacteria. *Annu. Rev. Phytopathol.* 30, 67–105.
- [7] Fett, W.F., Osman, S.F., Fishman, M.L. and Siebles, T.S. (1986) Alginate production by plant-pathogenic pseudomonads. *Appl. Environ. Microbiol.* 52, 466–473.

- [8] Osman, S.F., Fett, W.F. and Fishman, H.L. (1986) Exopolysaccharides of the phytopathogen *Pseudomonas syringae* pv. *glycinea*. J. Bacteriol. 166, 66–71.
- [9] Fett, W.F. and Dunn, M.F. (1989) Exopolysaccharides produced by phytopathogenic *Pseudomonas syringae* pathovars in infected leaves of susceptible hosts. Plant Physiol. 89, 5–9.
- [10] Gross, M. and Rudolph, K. (1987) Demonstration of levan and alginate in bean plants (*phaseolus vulgaris*) infected by *Pseudomonas syringae* pv. *phaseolicola*. J. Phytopathol. 120, 9–19.
- [11] Singh, S., Koehler, B. and Fett, W.F. (1992) Effect of osmolarity and dehydration on alginate production by fluorescent pseudomonads. Curr. Microbiol. 25, 335–339.
- [12] May, T.B., Shinabarger, D., Maharaj, K., Kato, J., Chu, L., DeVault, J.D., Roychaudhury, S., Zelinski, N.A., Berry, A., Rothmel, R.K., Misra, T.K. and Chakrabarty, A.M. (1991) Alginate synthesis by *Pseudomonas aeruginosa*: a key pathogenic factor in chronic pulmonary infections of cystic fibrosis patients. Clin. Microbiol. Rev. 4, 91–206.
- [13] Bruegger, B.B. and Kenn, N.T. (1979) Specific elicitors of glyceollin accumulation in the *Pseudomonas glycinea* soybean host-parasite system. Physiol. Plant Pathol. 15, 43–51.
- [14] Blumenkrantz, N. and Asboe-Hansen G. (1973) New method for quantitative determination of uronic acids. Anal. Biochem. 54, 484–489.
- [15] Dubois, M., Giles, K.A., Hamilton, G.K., Rebeos, P.A. and Smith, F. (1956) Colorimetric method of determination of sugars and related substances. Anal. Chem. 28, 350–356.
- [16] Ashwell, G. (1966) New colorimetric method for sugar analysis. Methods in Enzymology Vol. VII (Neufeld, E.F. and Ginsberg, V., Eds.), pp. 85–95, Academic Press, New York, NY.
- [17] Varma, R., Varma, R.S. and Wardi, A.H. (1973) Separation of aldononitrile acetates of neutral sugars by gas-liquid chromatography and its application to polysaccharides. J. Chromatogr. 77, 222–227.
- [18] Zevenhuisen, L.P.T.M. and Faleschini, P. (1991) Effect of the concentration of sodium chloride in the medium on the relative proportions of poly- and oligo-saccharides excreted by *Rhizobium meliloti* strain YE-2SL. Carbohydr. Res. 209, 203–209.
- [19] Ramm, M. (1991) Occurrence of *Pseudomonas syringae* pv. *phaseolicola* lipopolysaccharide in the culture medium and their possible functions in pathogenesis. In: Proceedings of the 4th International Working Group on *Pseudomonas syringae* Pathovars (Durbin, R.D., Surico, G. and Mugnai, L., Eds.), pp. 74–81.
- [20] Zdorovenko, G.M., Gubanova, N.Ya., Solyanik, L.P., Knirel, Yu.A., Yakovleva, L.M. and Zakarova, I.Ya. (1991) Composition and structure of lipopolysaccharide from the strains of different pathovars of *Pseudomonas syringae*. In: Proceedings of the 4th International Working Group on *Pseudomonas syringae* pathovars (Durbin, R.D., Surico, G. and Mugnai, L., Eds.), pp. 391–401.
- [21] Vinogradov, E.V., Shashkov, A.S., Krirel, V.A., Zdorovenko, G.H., Solyanik, L.P., Gubanova, N.Y. and Yakovleva, L.M. (1991) Somatic antigens of the pseudomonads: structure of the O-specific polysaccharide chain of *Pseudomonas syringae* pv. *tabaci* 225 (serogroup VIII) lipopolysaccharide. Carbohydr. Res. 212, 307–311.
- [22] Liao, C.-H., McCallus, D.E. and Fett, W.F. (1994) Molecular characterization of two gene loci required for production of the key pathogenicity factor pectate lyase in *Pseudomonas viridiflava*. Mol. Plant-Microbe Interact. 7, 391–400.
- [23] Allison, D.G. and Goldsbrough, M.J. (1994) Polysaccharide production in *Pseudomonas cepacia*. J. Basic Microbiol. 34, 3–10.
- [24] Xiao, Y., Lu, Y., Heu, S. and Hutcheson, S.W. (1992) Organization and environmental regulation of the *Pseudomonas syringae* pv. *syringae* 61 *hrp* cluster. J. Bacteriol. 175, 1767–1776.